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Title: **Method for Separating Cells Using Immunorosettes**

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This application is a continuation-in-part of United States application no. 09/579,463 filed May 26, 2000 which claims benefit from United States provisional application serial no. 60/203,477 filed on May 11, 2000; United States provisional application serial no. 60/193,371 filed on March 31, 2000 (now abandoned); and United States provisional application no. 60/136,770 filed on May 28, 1999 (now abandoned), all of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to methods for separating cells using immunorosettes. The invention includes novel antibody compositions for use in the method of the invention.

BACKGROUND OF THE INVENTION

In many applications it is desirable to enrich or, alternatively, deplete certain cell populations in a biological sample. The fields of hematology, immunology and oncology rely on samples of peripheral blood and cell suspensions from related tissues such as bone marrow, spleen, thymus and fetal liver. The separation of specific cell types from these heterogeneous samples is key to research in these fields, diagnostics and therapy for certain malignancies and immune/hematopoietic disorders.

Purified populations of immune cells such as T cells and antigen presenting cells are necessary for the study of immune function and are used in immunotherapy. Investigation of the cellular, molecular and biochemical processes require analysis of certain cell types in isolation. Numerous techniques have been used to isolate T cell subsets, B cells, basophils, NK cells and dendritic cells.

The isolation of hematopoietic stem cells has also been an area of great interest. Pure populations of stem cells will facilitate studies of hematopoiesis and transplantation of hematopoietic cells from peripheral blood and/or bone marrow is increasingly used in combination with high-dose chemo- and/or radiotherapy for the treatment of a variety of disorders

including malignant, nonmalignant and genetic disorders. Very few cells in such transplants are capable of long-term hematopoietic reconstitution, and thus there is a strong stimulus to develop techniques for purification of hematopoietic stem cells. Furthermore, serious complications and indeed the success of a transplant procedure is to a large degree dependent on the effectiveness of the procedures that are used for the removal of cells in the transplant that pose a risk to the transplant recipient. Such cells include T lymphocytes that are responsible for graft versus host disease (GVHD) in allogenic grafts, and tumor cells in autologous transplants that may cause recurrence of the malignant growth. It is also important to debulk the graft by removing unnecessary cells and thus reducing the volume of cyropreservant to be infused.

In certain instances it is desirable to remove or deplete tumor cells from a biological sample, for example in bone marrow transplants. Epithelial cancers of the bronchi, mammary ducts and the gastrointestinal and urogenital tracts represent a major type of solid tumors seen today.

Micrometastatic tumor cell migration is thought to be an important prognostic factor for patients with epithelial cancer (Braun et al., 2000; Vaughan et al., 1990). The ability to detect such metastatic cells is limited by the effectiveness of tissue or fluid sampling and the sensitivity of tumor detection methods. A technique to enrich circulating epithelial tumor cells in blood samples would increase the ability to detect metastatic disease and facilitate the study of such rare cells and the determination of the biological changes which enable spread of disease.

Hematopoietic cells and immune cells have been separated on the basis of physical characteristics such as density and on the basis of susceptibility to certain pharmacological agents which kill cycling cells. The advent of monoclonal antibodies against cell surface antigens has greatly expanded the potential to distinguish and separate distinct cell types. There are two basic approaches to separating cell populations from blood and related cell suspensions using monoclonal antibodies. They differ in whether it is the desired or undesired cells which are distinguished/labelled with the antibody(s).

In positive selection techniques the desired cells are labelled with antibodies and removed from the remaining unlabelled/unwanted cells. In negative selection, the unwanted cells are labelled and removed. Antibody/complement treatment and the use of immunotoxins are negative selection techniques, but FACS sorting and most batch wise immunoadsorption techniques can be adapted to both positive and negative selection. In immunoadsorption techniques cells are selected with monoclonal antibodies and preferentially bound to a surface which can be removed from the remainder of the cells e,g. column of beads, flasks, magnetic particles. Immunoadsorption techniques have won favour clinically and in research because they maintain the high specificity of targeting cells with monoclonal antibodies, but unlike FACSorting, they can be scaled up to deal directly with the large numbers of cells in a clinical harvest and they avoid the dangers of using cytotoxic reagents such as immunotoxins, and complement. They do however, require the use of a "device" or cell separation surface such as a column of beads, panning flask or magnet.

Current techniques for the isolation of hematopoietic stem cells, immune cells and circulating epithelial tumor cells all involve an initial step to remove red cells then antibody mediated adherence to a device or artificial particle. (Firat et al., 1988; de Wynter et al., 1975; Shpall et al., 1994; Thomas et al., 1994; Miltenyi Biotec Inc., Gladbach, Germany) In the case of positive selection there is yet another step; removal of the cells from the device or particle. All these multiple steps require time and incur cell loss. Slaper-Cortenbach et al. (1990) describes a method for purging bone marrow of common acute leukemic (cALL) cells using immunorosetting. The method requires that the erythrocytes are first removed from the bone marrow sample and are labelled with antibodies that bind to the cALL cells. The labelled erythrocytes are then added back to the sample where the cALL cells are immunorosetted. The depletion method works best when followed by an additional step of complement mediated lysis of the cALL cells.

Density Separations are commonly used to isolate peripheral blood mononuclear cells from granulocytes and erythrocytes. Ficoll (Amersham Pharmacia Biotech AB, Uppsala Sweden) is the most popular

density solution used for this application. In a Ficoll density separation whole blood is layered over Ficoll, and then centrifuged. The erythrocytes and granulocytes settle to the cell pellet and the mononuclear cells remain at the Ficoll plasma interface. The success of this technique relies on the difference in density between mononuclear cells and granulocytes. If whole blood is stored for more than 24 hours the granulocytes change density and will not pellet with the red cells. Suspensions of pure mononuclear cells can not be obtained from stored blood or samples with altered cell density in a single density separation.

In view of the foregoing, there is a need in the art to provide novel methods for separating desired cells or removing unwanted cells from biological samples.

SUMMARY OF THE INVENTION

The present inventors have developed a method for separating cells by immunorosetting the cells with red blood cells or erythrocytes already existing in the sample. The method of the invention is a much simpler yet equally efficient immunoaffinity technique as compared to the prior art methods. There is no "device" or need for an artificial separation surface (e.g., magnetic particles, affinity column) not normally present in the cell suspension. There is no need to first remove the erythrocytes from the sample and to then re-introduce them once they have been labelled with antibodies. Specific cell types are cross-linked to autologous erythrocytes found within the sample and subsequent rosettes are then removed by sedimentation or centrifugation.

Accordingly, in one embodiment, the present invention provides a method of separating nucleated cells from a sample comprising the nucleated cells and erythrocytes comprising:

(1) contacting the sample with an antibody composition comprising (a) at least one antibody that binds to an antigen on the nucleated cells to be separated linked, either directly or indirectly, to (b) at least one antibody that binds to the erythrocytes, under conditions to allow immunorosettes of the nucleated cells and the erythrocytes to form; and

(2) removing the immunorosettes from the sample.

The method can be used in both positive and negative selection protocols. The method can be used on any sample that contains red blood cells including whole blood, bone marrow, fetal liver, cord blood, buffy coat suspensions, pleural and peritoneal effusion and samples of thymocytes and splenocytes.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 is a schematic diagram of a rosette of erythrocytes formed around an unwanted nucleated cell using tetrameric antibody complexes.

DETAILED DESCRIPTION OF THE INVENTION

I. METHOD OF THE INVENTION

As hereinbefore mentioned, the present invention relates to a method for separating cells by immunorosetting the cells with red blood cells.

In its broadest aspect, the present invention provides a method of separating nucleated cells from a sample comprising the nucleated cells and erythrocytes comprising:

(1) contacting the sample with an antibody composition comprising (a) at least one antibody that binds to an antigen on the nucleated cells to be separated linked, either directly or indirectly, to (b) at least one antibody that binds to the erythrocytes, under conditions to allow immunorosettes of the nucleated cells and the erythrocytes to form; and

(2) removing the immunorosettes from the sample.

The method can be used in both positive and negative selection protocols. In positive selection, the desired cells are rosetted. In such an embodiment, the method would further include the step of lysing the red blood cells in the immunorosettes and separating the desired cells. Accordingly, in a positive selection method the antibody composition will contain (a) at least one antibody specific for the nucleated cells that one wishes to obtain or separate from the sample.

Preferably, the method of the invention is used in a negative selection protocol. In negative selection, the desired cells are not immunorosetted and would be remaining in the sample once the immunorosettes have been removed. In a negative selection method, the antibody composition will contain (a) at least one antibody specific for the cells that one wishes to remove from the sample. Accordingly, the present invention provides a negative selection method for enriching and recovering desired cells in a sample containing the desired cells, erythrocytes and undesired cells comprising:

(1) contacting the sample with an antibody composition comprising (a) at least one antibody that binds to an antigen on the undesired cells linked, either directly or indirectly, to (b) at least one antibody that binds to the erythrocytes, under conditions to allow immunorosettes of the undesired cells and the erythrocytes to form; and

(2) separating the immunorosettes from the remainder of the sample to obtain a sample enriched in the desired cells.

The immunorosettes between the erythrocytes and the unwanted cells formed in step (1) can be separated from the desired cells using a variety of techniques. In one embodiment, the sample, containing the immunorosettes, is layered over a buoyant density solution (such as Ficoll-Hypaque) and centrifuged. The immunorosettes pellet and the desired cells remain at the interface between the buoyant density solution and the sample. The desired cells are then removed from the interface for further use. In another embodiment, the sample containing the immunorosettes obtained in step (1) is mixed with a sedimentation reagent (such as hydroxyethyl starch, gelatin or methyl cellulose) and the rosettes are permitted to sediment. The desired cells remain in suspension and are removed for further use. In a further embodiment, the sample containing the immunorosettes obtained in step (1) is allowed to sediment with or without the aid of centrifugation or Counter Flow Elutriation. The desired cells remain in suspension and are removed for further use.

The antibody compositions for use in the present invention are described in greater detail below.

The method of the invention may be used in the processing of biological samples that contain erythrocytes including blood (in particular, cord blood and whole blood) bone marrow, fetal liver, buffy coat suspensions, pleural and peritoneal effusions and suspensions of thymocytes and splenocytes. Surprisingly, the inventors have found that the method can be used to remove cells directly from whole blood or whole bone marrow without prior processing. This offers a significant advantage of the method of the invention over the prior art methods. In particular, the erythrocytes do not have to be removed, labelled and added back to the sample.

The method of the invention can be used to prepare enriched samples of any cell type including, but not limited to, T cells, B cells, NK cells, dendritic cells, monocytes, basophils, mast cells, progenitor cells, stem cells and tumor cells.

In one embodiment, the method of the invention can be used to enrich for non-hematopoietic cells including normal cells as well as non-hematopoietic tumor cells. Examples of normal non-hematopoietic cells include epithelial cells, endothelial cells, mesenchymal cells or precursors thereof.

In yet another embodiment, the method of the invention can be used to separate non-hematopoietic tumor cells, such as non-hematopoietic metastatic tumor cells from a sample. The method is useful in the detection of non-hematopoietic tumor cells from blood, bone marrow, and peritoneal and pleural effusions of patients to aid in the diagnosis and detection of metastatic disease, monitoring the progression of metastatic disease, or monitoring the efficacy of a treatment.

In another embodiment, the method of the invention can be used to prepare hematopoietic progenitor and stem cell preparations from bone marrow samples. For example, the method of the invention may be used in a negative selection protocol to deplete or purge B and T lymphocytes, monocytes, NK cells, granulocytes, and/or tumor cells from samples to prepare hematopoietic progenitor and stem cell preparations for

use in transplantation as well as other therapeutic methods that are readily apparent to those of skill in the art. For example, bone marrow or blood can be harvested from a donor in the case of an allogenic transplant and enriched for progenitor and stem cells by the method described herein. Using negative selection the human hematopoietic progenitor and stem cells in the preparation are not coated with antibodies, or modified making them highly suitable for transplantation and other therapeutic uses that are readily apparent to those skilled in the art.

In another embodiment, the method of the invention can be used to isolate and recover mature dendritic cells and their precursors from blood. Dendritic cells have many useful applications including as antigen presenting cells capable of activating T cells both in vitro and in vivo. As an example, dendritic cells can be loaded (pulsed) in vitro with a tumor antigen and injected in vivo to induce an anti-tumor T cell response.

In a further embodiment, the method of the invention may also be used to prepare a cell preparation from samples such as blood and bone marrow, which is enriched in a selected differentiated cell type such as T-cells, B-cells, NK cells, monocytes, dendritic cells, basophils and plasma cells. This will enable studies of specific cell to cell interactions including growth factor production and responses to growth factors. It will also allow molecular and biochemical analysis of specific cells types. Cell preparations enriched in NK cells, dendritic cells and T-cells may also be used in immune therapy against certain malignancies.

II. ANTIBODY COMPOSITIONS

The invention includes the antibody compositions for use in the method of the present invention. The antibody composition will contain (a) at least one antibody that binds to an antigen on nucleated cells linked, either directly or indirectly, to (b) at least one antibody that binds to an antigen on erythrocytes.

The term "at least one antibody" means that the antibody composition includes at least one type of antibody (as opposed to at least one antibody molecule). One type of antibody means an antibody that binds to a particular antigen. For example, antibodies that bind to the antigen CD2 are

considered one type of antibody. Preferably, the antibody compositions of the invention contain (a) more than one antibody type that binds to nucleated cells.

The two antibodies (a) and (b) may be directly linked by preparing bifunctional or bispecific antibodies. The two antibodies (a) and (b) may be indirectly linked for example, by preparing tetrameric antibody complexes. All of these are described hereinafter.

In one aspect, the antibody specific for the nucleated cells is linked directly to the antibody specific for the erythrocytes. In one embodiment, the antibody composition of the present invention contains bifunctional antibodies comprising at least one antibody specific for the nucleated cells linked directly to (b) at least one antibody specific for the erythrocytes. Bifunctional antibodies may be prepared by chemically coupling one antibody to the other, for example by using N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP).

In another embodiment, the antibody composition contains bispecific antibodies. Bispecific antibodies contain a variable region of an antibody specific for erythrocytes and a variable region specific for at least one antigen on the surface of the nucleated cells to be separated. The bispecific antibodies may be prepared by forming hybrid hybridomas. The hybrid hybridomas may be prepared using the procedures known in the art such as those disclosed in Staerz & Bevan, (1986, PNAS (USA) 83: 1453) and Staerz & Bevan, (1986, Immunology Today, 7:241). Bispecific antibodies may also be constructed by chemical means using procedures such as those described by Staerz et al., (1985, Nature, 314:628) and Perez et al., (1985 Nature 316:354), or by expression of recombinant immunoglobulin gene constructs.

In another aspect, the antibody composition of the present invention comprises (a) at least one antibody specific for a nucleated cell type indirectly linked to (b) at least one antibody specific for the erythrocyte. By "indirectly linked" it is meant that antibody (a) and antibody (b) are not directly covalently linked to each other but are attached through a linking moiety such as an immunological complex. In a preferred embodiment, the

antibody to the nucleated cell type is indirectly linked to the antibody specific for the erythrocytes by preparing a tetrameric antibody complex. A tetrameric antibody complex may be prepared by mixing a first monoclonal antibody which is capable of binding to the erythrocytes, and a second monoclonal antibody capable of binding the nucleated cells to be separated. The first and second monoclonal antibody are from a first animal species. The first and second antibody are reacted with approximately an equimolar amount of monoclonal antibodies of a second animal species which are directed against the Fc-fragments of the antibodies of the first animal species. The first and second antibody may also be reacted with an about equimolar amount of the F(ab')2 fragments of monoclonal antibodies of a second animal species which are directed against the Fc-fragments of the antibodies of the first animal species. (See U.S. Patent No. 4,868,109 to Lansdorp, which is incorporated herein by reference for a description of tetrameric antibody complexes and methods for preparing same).

Preferably, the antibody specific for the erythrocytes is anti-glycophorin A. The anti-glycophorin A antibodies contained in the antibody composition of the invention are used to bind the erythrocytes. Examples of monoclonal antibodies specific for glycophorin A are 10F7MN (U.S. Patent No. 4,752,582, Cell lines: ATCC accession numbers HB-8162), and D2.10 (Immunotech, Marseille, France).

Preferably, the antibody specific for the nucleated cells is a combination of antibodies. The combination of antibodies may be specific for a number of cell types so that many cell types may be removed from the sample. When using a combination of antibodies, each antibody will be linked (either directly or indirectly) to an antibody specific for erythrocytes.

In a preferred embodiment, the antibody composition is a tetrameric complex comprising (a) anti-glycophorin A antibodies to bind the erythrocytes, (b) an antibody that binds to a nucleated cell type that one wishes to immunorosette and (c) antibodies that bind the Fc portion of both (a) and (b), optionally F(ab')2 antibody fragments. The molar ratio of (a):(b):(c) may be approximately 1:3:4. When several types of cells are to be separated, complexes are made with several anti-nucleated cell antibodies (b).

The complexes may then be mixed together to form an antibody composition for use in the method of the invention. Figure 1 is a schematic diagram of a rosette formed by tetrameric antibody complexes.

Within the context of the present invention, antibodies are understood to include monoclonal antibodies and polyclonal antibodies, antibody fragments (e.g., Fab, and F(ab')2), chimeric antibodies, bifunctional or bispecific antibodies and tetrameric antibody complexes. Antibodies are understood to be reactive against a selected antigen on the surface of a nucleated cell or erythrocyte if they bind with an appropriate affinity (association constant), e.g. greater than or equal to 107 M-1.

Monoclonal antibodies are preferably used in the antibody compositions of the invention. Monoclonal antibodies specific for selected antigens on the surface of nucleated cells may be readily generated using conventional techniques. For example, monoclonal antibodies may be produced by the hybridoma technique originally developed by Kohler and Milstein 1975 (Nature 256, 495-497; see also U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993 which are incorporated herein by reference; see also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Other techniques may also be utilized to construct monoclonal antibodies (for example, see William D. Huse et al., 1989, "Generation of a Large Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281, L. Sastry et al., 1989 "Cloning of the Immunological Repertoire in Escherichia coli for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain Variable Region-Specific cDNA Library," Proc Natl. Acad. Sci USA 86:5728-5732; Kozbor et al., 1983 Immunol. Today 4, 72 re the human B-cell hybridoma technique; Cole et al. 1985 Monoclonal Antibodies in Cancer Therapy, Allen R. Bliss, Inc., pages 77-96 re the EBV-hybridoma technique to produce human monoclonal antibodies; and see also Michelle Alting-Mees et al., 1990 "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas," Strategies in Molecular Biology 3:1-9). Hybridoma cells can be

screened immunochemically for production of antibodies specifically reactive with an antigen, and monoclonal antibodies can be isolated.

Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')2 fragments can be generated by treating antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

The invention also contemplates chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. A variety of approaches for making chimeric antibodies have been described and can be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes selected antigens on the surface of differentiated cells or tumor cells. See, for example, Morrison et al., 1985; Proc. Natl. Acad. Sci. U.S.A. 81,6851; Takeda et al., 1985, Nature 314:452; Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom patent GB 2177096B.

Bifunctional antibodies may be prepared by chemical conjugation, somatic hybridization or genetic engineering techniques.

Chemical conjugation is based on the use of homo- and heterobifunctional reagents with e-amino groups or hinge region thiol groups. Homobifunctional reagents such as 5,5'-Dithiobis(2-nitrobenzoic acid)(DNTB) generate disulfide bonds between the two Fabs, and 0-phenylenedimaleimide (O-PDM) generate thioether bonds between the two Fabs (Brenner et al., 1985, Glennie et al., 1987). Heterobifunctional reagents such as N-succinimidyl-3-(2-pyridylditio) propionate (SPDP) combine exposed amino groups of antibodies and Fab fragments, regardless of class or isotype (Van Dijk et al., 1989).

Somatic hybridization includes fusion of two established hybridomas generating a quadroma (Milstein and Cuello, 1983) or fusion of

one established hybridoma with lymphocytes derived from a mouse immunized with a second antigen generating a trioma (Nolan and Kennedy, 1990). Hybrid hybridomas are selected by making each hybridoma cell line resistant to a specific drug-resistant marker (De Lau et al., 1989), or by labeling each hybridoma with a different fluorochrome and sorting out the heterofluorescent cells (Karawajew et al., 1987).

Genetic engineering involves the use of recombinant DNA based technology to ligate sequences of DNA encoding specific fragments of antibodies into plasmids, and expressing the recombinant protein. Bispecific antibodies can also be made as a single covalent structure by combining two single chains Fv (scFv) fragments using linkers (Winter and Milstein, 1991); as leucine zippers coexpressing sequences derived from the transcription factors fos and jun (Kostelny et al., 1992); as helix-turn-helix coexpressing an interaction domain of p53 (Rheinnecker et al., 1996), or as diabodies (Holliger et al., 1993).

Table 1 provides examples of antibodies to particular human antigens on nucleated cells that may be used in the method of the present invention. The method of the invention may also be used for other species. The choice of the antibody or antibodies to the nucleated cells will depend on the nature of the sample, the choice of the cells to be enriched or depleted and whether the method is a positive or negative selection protocol. In all cases, the antibody (or antibodies) to the nucleated cells to be immunorosetted will be linked, either directly or indirectly, to the antibody specific for the erythrocytes when used in the method of the invention.

The methods and antibody compositions of the invention are preferably used in negative selection protocols to prepare a cell preparation which is enriched for a specific cell type. This is achieved by using antibody compositions which lack antibodies to the specific cell type that you wish to isolate. Accordingly, the present invention provides an antibody composition for enriching and recovering desired cells in a sample containing desired cells, erythrocytes and undesired cells comprising (a) at least one antibody that binds to an antigen on the undesired cells linked to (b) at least one antibody that binds to the erythrocytes. Particular embodiments of the antibody

compositions that may be used in negative selection protocols of the invention for human cells are set out in Table 2. This Table provides a list or cocktail of antibodies to particular antigens that can be used as antibody (a) in the above method to enrich for a particular cell type. In most cases, several choices for the essential antibodies are provided as well as several optional antibodies. For example, for enriching for T cells antibody (a) may be a cocktail of antibodies to (1) CD16 and/or CD66b and/or CD11b and/or CD15; (2) CD19 and/or CD20 and/or CD21 and/or CD22 and/or CD24 and/or Ig; and (3) CD36 and/or CD14. The cocktail may optionally include antibodies to CD33 and/or CD56 and/or IgE and/or CD41. As another example, for enriching for non-hematopoietic cells antibody (a) may be a cocktail of antibodies to (1) CD45 and (2) CD66b and optionally CD36 and/or CD2 and/or CD3 and/or CD14 and/or CD16 and/or CD19 and/or CD38 and/or CD56 and/or CD66e. In a specific embodiment, the antibody composition for enriching for non-hematopoietic tumor cells comprises antibodies to CD45, CD66b and CD36 in addition to the antibodies that can bind the erythrocytes. In addition to the antibody combinations listed in Table 2, one skilled in the art will appreciate that other antibody combinations may be used to enrich for specific cell types such as those described in U.S. Patent No. 5,877,299 which is incorporated herein by reference. As the invention relates to the preparation of immunorosettes to prepare enriched cell preparations, one skilled in the art will appreciate that other antibodies and antibody combinations may be used.

The methods and antibody compositions of the invention may be used in positive selection protocols to prepare a cell preparation in which the desired cells are immunorosetted. Some examples of antibody combinations useful in positive selection protocols are set out below.

To separate non-hematopoietic tumor cells in a positive selection protocol, the antibody composition includes antibodies specific for non-hematopoietic antigens expressed on tumor cells, such as antibodies against antigens expressed on the surface of breast and lung carcinoma and neuroblastoma cells. The antibodies to the non-hematopoietic antigens expressed on epithelial tumor cells may be obtained from commercial sources

(for example as shown in Table 3) or prepared using techniques known in the art.

To separate B cells in a positive selection protocol, the antibody composition contains antibodies against CD24 and/or CD19 and/or CD20 and/or CD22.

To separate T cells in a positive selection protocol, the antibody composition contains antibodies against CD3 and/or CD2 and/or CD5 and/or both CD4 and CD8.

To separate NK cells in a positive selection protocol, the antibody composition contains antibodies against CD56.

To separate granulocytes in a positive selection protocol, the antibody composition contains antibodies against CD16 and/or CD66e and/or CD66b.

To separate monocytes in a positive selection protocol, the antibody composition contains antibodies against CD14.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1

Preparation of Tetramers

In order to prepare a tetrameric antibody complex for use in the method of the present invention, the following protocol may be used: (a) take 1 mg of antibody specific for cells to be rosetted (e.g. anti-CD2, CD3, CD4, CD8, CD14, CD16, CD19 etc.); (b) add 3 mg anti-Glycophorin A antibody (against red blood cells); mix well (c) then add 4.0 mg of P9 antibody or 2.72 mg of the P9 F(ab')2 antibody fragment. Incubate overnight at 37°C. The P9 antibody binds the Fc portion of the antibodies added in steps (a) and (b) resulting in a tetrameric antibody complex. For more information on the preparation of tetramers see U.S. Patent No. 4,868,109 to Lansdorp, which is incorporated herein by reference. Tetrameric antibody complexes incorporating different antibodies to antigens expressed or nucleated cells are prepared separately and then mixed.

The antibody compositions are made by combining various tetrameric antibody complexes depending on which cells one wishes to deplete. The concentration of the various tetrameric antibody complexes varies: typically antibodies to antigens expressed on nucleated cells are at 10-30mg/mL in tetrameric complexes. The composition is then diluted 1/10 into the cells so the final concentrations of each anti nucleated cell antibody in the cell suspensions is 1.0-3.0 mg/mL.

Example 2

Method of Immunorosetting Using Ficoll

A negative selection protocol for immunorosetting cells from whole peripheral blood using Ficoll Hypaque is set out below.

1. Add 100mL antibody composition per mL of whole peripheral blood.
2. Incubate 20 minutes at room temperature.
3. Dilute sample with an equal volume of phosphate buffered saline (PBS) + 2% fetal calf serum (FCS) and mix gently.
4. Layer the diluted sample on top of Ficoll Hypaque or layer the Ficoll underneath the diluted sample.
5. Centrifuge for 20 minutes at 1200 x g, room temperature, with the brake off.
6. Remove the enriched cells from the Ficoll:plasma interface.
7. Wash enriched cells with 5-10x volume of PBS + 2% FBS.

Note: For enrichment of monocytes and other adherent cells, add 1 mM EDTA to the sample of whole blood and to all wash/dilution solutions.

Example 3

Method of Immunorosetting Using Hetastarch Sedimentation

A negative selection protocol for immunorosetting cells from whole peripheral blood using hetastarch is set out below. Hetastarch is one of a number of compounds that increases red blood cell sedimentation rates through agglutination.

1. Add 1 mL of 6% hetastarch in saline per 5 mL of blood and mix.

2. Add antibody composition described in Example 1 to whole blood such that each anti-nucleated cell antibody is at a final concentration of 1.0-2.0 mg/mL.
3. Incubate 10 minutes at room temperature.
4. Centrifuge for 5 minutes at 50 x g, room temperature.
5. Remove supernatant. This fraction contains the enriched cells.
6. Wash enriched cell fraction with 2-5 x volume of PBS +2% fetal bovine serum (FBS).

Example 4

Method of Immunorosetting Using Hetastarch/Iodixanol Mixture

A negative selection protocol for immunorosetting cells from whole peripheral blood is set out below.

1. Add 1 mL of 6% hetastarch in saline per 5 mL of blood and mix.
2. Add 0.6 mL of 60% w/v iodixanol and mix. Iodixanol is one of a number of compounds that increases the aqueous solution density appreciably.
3. Add antibody composition described in Example 1 to whole blood such that each anti-nucleated cell antibody is at a final concentration of 1.0-2.0 mg/mL.
4. Incubate 10 minutes at room temperature.
5. Centrifuge for 5 minutes at 50 xg, room temperature.
6. Remove supernatant. This fraction contains the enriched cells.
7. Wash enriched cell fraction with 2-5 x volume of PBS +2% FBS.

Example 5

Method of Immunorosetting - Positive Selection

A positive selection protocol for immunorosetting cells from whole peripheral blood is set out below.

1. Set aside 1 mL of blood.

2. Layer 10 mL of blood over Ficoll-Paque and centrifuge for 20 minutes at 1200xg, room temperature, brake off.
3. Recover the MNC layer at the Ficoll:plasma interface, wash with PBS+2% FBS.
4. Count cells and resuspend at 1x108/mL.
5. Measure sample volume, designated volume A.
6. Add 0.2 mL of reserved blood from Step 1.
7. Make up total volume to twice volume A with PBS+2% FBS.
8. Add a tetrameric antibody complex specific to a given antigen at a final concentration of 1.0 mg/mL, the synthesis of which is described in Example 1.
9. Incubate 20 minutes at room temperature.
10. Dilute by a factor of 2 with PBS+2% FBS and layer over Percoll prepared at a density of 1.085 g/mL and an osmolarity of 280 mOsm.
11. Centrifuge for 20 minutes at 1200xg as in Step 2.
12. Discard supernatant and resuspend pellet containing the enriched cells.
13. Lyse red blood cells with ammonium chloride solution and wash with PBS+2% FBS.

Example 6

Enrichment of T cells - Immunorosetting Using Ficoll

This example demonstrates the enrichment of T cells from whole peripheral blood using the method described in Example 2. A T cell enrichment cocktail of tetrameric antibody complexes containing antibodies against CD16, CD19, CD36 and CD56 was prepared. The results, shown in Table 4, demonstrate that the method of the invention results in 95% purity of T cells with a recovery of close to 50%.

Example 7

Enrichment of CD8+ T cells - Immunorosetting Using Ficoll

This example demonstrates the enrichment of CD8+ T cells from whole peripheral blood using the method described in Example 2. Two cocktails of tetrameric antibody complexes were tested. One cocktail

contained antibodies against CD4, CD16, CD19, CD36 and CD56 the other contained antibodies to CD4, CD16, CD19, CD36, CD56 and IgE. The results, shown in Table 5, demonstrate that the addition of anti IgE to the cocktail improves the purity of CD8+ T cells with no effect on recovery.

Example 8

Enrichment of CD4+ T cells - Immunorosetting Using Ficoll

This example demonstrates the enrichment of CD4+ T cells from whole peripheral blood using the method described in Example 2. Two CD4 T cell enrichment cocktails of tetrameric antibody complexes were prepared. One cocktail contained antibodies to CD8, CD16, CD19, CD36 and CD56. The other cocktail contained antibodies to CD8, CD16, CD19, CD36, CD56 and IgE. The results, shown in Table 6, demonstrate that the method of the invention results in 93% purity of CD4+ T cells with a recovery of 46% and that addition of anti-IgE to the enrichment cocktail improves the purity of CD4+ T cells.

Example 9

Enrichment of B cells - Immunorosetting Using Ficoll

This example demonstrates the enrichment of B cells from whole peripheral blood using the method described in Example 2. Two B cell enrichment cocktails of tetrameric antibody complexes were prepared. One cocktail contained antibodies to CD2, CD3, CD16, CD36 and CD56. The other cocktail contained antibodies to CD2, CD3, CD16, CD36, CD56 and IgE. The results, shown in Table 7, demonstrate that the method of the invention results in 88% purity of B cells with a recovery of 43% and that addition of anti-IgE to the cocktail improves the purity of B cells.

Example 10

Enrichment of NK cells - Immunorosetting Using Ficoll

This example demonstrates the enrichment of NK cells from whole peripheral blood using the method described in Example 2. Two NK cell enrichment cocktails of tetrameric antibody complexes were prepared. One cocktail contained antibodies to CD3, CD4, CD19, CD66b and CD36. The other cocktail contained antibodies to CD3, CD4, CD19, CD66b, CD36 and IgE. The results, shown in Table 8, demonstrate that the method of the

invention results in 74% purity of NK cells with a recovery of 44% and that the addition of anti-IgE to the cocktail improves purity but decreases recovery.

Example 11

Enrichment of Progenitors

This example demonstrates the enrichment of progenitor cells from whole umbilical cord blood using the method described in Example 2. Two different cocktails of tetrameric antibody complexes were used;

(a) the progenitor enrichment cocktail containing tetrameric antibody complexes to CD2, CD3, CD14, CD16, CD19, CD24, CD56 and CD66b;

(b) the de-bulking cocktail containing tetrameric antibody complexes to CD2, CD14, CD19 and CD66b.

The results, shown in Table 9, demonstrate that the method of the invention results in 29% purity of CD34+ cells with a recovery of 53% for the extensive progenitor enrichment cocktail and only 5% purity and 45% recovery for the four antibody de-bulking cocktail.

Example 12

Enrichment of Monocytes - Immunorosetting Using Ficoll

This example demonstrates the enrichment of monocytes from whole peripheral blood using the method described in Example 2. Several monocyte enrichment cocktails of tetrameric antibody were prepared (see Table 10). The results shown in Table 10 demonstrate that the method of the invention results in 76% purity of CD14+ cells with 65% recovery of CD14+ cells and that the addition of anti CD8 or anti-IgE improved the purity of monocytes but adding both anti-CD8 and IgE did not have an additive effect.

Example 13

Enrichment of Non-Hematopoietic Tumor Cells

This example demonstrates the enrichment of breast cancer cells from whole peripheral blood using the method described in Example 2. Cells from the CAMA breast cancer cell line were seeded into samples of whole peripheral blood at a frequency of 1/103, 1/104 and 1/105. Four tumor cell enrichment cocktails of tetrameric antibody complexes were prepared. The

antibody composition of the cocktails is listed in Table 11. The results, shown in Table 12, demonstrate that the method of the invention results in greater than 2 log enrichment of tumor cells with 20-50% recovery of tumor cells. The more extensive cocktail offers a greater degree of tumor cell enrichment.

The results shown in Table 13, demonstrate the effect of adding anti-CD36 to the tumor enrichment cocktails. Cells from the CAMA breast cancer cell line were seeded into samples of previously frozen peripheral blood mononuclear cell to a concentration of 1.4 CAMA cells per 1,000 nucleated cells. Addition of anti-CD36 to a cocktail containing anti-CD45 and anti-CD66b improved the log enrichment of CAMA cells from 2.6 log to 2.7 log and the purity of CAMA cells from 58% to 77%. However, neither the log enrichment or purity reached the levels attained with the extensive cocktail.

Example 14

T Cell Enrichment - Effect of Substituting Anti-CD14 with Anti-CD36

This example demonstrates the improved T cell enrichment from whole peripheral blood using the method described in Example 2 when the enrichment cocktail is modified by substituting anti-CD36 for anti-CD14. The results in Table 14 show a 24% increase in %purity of CD3+ cells with the antibody substitution.

Example 15

Enrichment of Specific Cell Populations Using Hetastarch Sedimentation

This example demonstrates the enrichment of various cell populations from whole peripheral blood using the method described in Example 3.

A T cell enrichment cocktail of tetrameric antibody complexes containing antibodies against CD16, CD19, CD36 and CD56 was prepared. The method of the invention results in greater than 95% purity of T cells, with a recovery of 60%.

A B cell enrichment cocktail of tetrameric antibody complexes containing antibodies against CD2, CD3, CD16, CD36 and CD56 was prepared. The method of the invention results in 75% purity of B cells, with a recovery of 39%.

A NK cell enrichment cocktail of tetrameric antibody complexes containing antibodies against CD3, CD4, CD19, CD36 and CD66b was prepared. The method of the invention results in 65% purity of NK cells, with a recovery of 27%.

Example 16

Enrichment of Specific Cell Populations Using Hetastarch/Iodixanol Mixture

This example demonstrates the enrichment of various cell populations from whole peripheral blood using the method described in Example 4. The results, listed in Table 15, are summarized as follows.

A T cell enrichment cocktail of tetrameric antibody complexes containing antibodies against CD16, CD19, CD36 and CD56 was prepared. The method of the invention results in 95% purity of T cells, with a recovery of 61%.

A CD4+ T cell enrichment cocktail of tetrameric antibody complexes containing antibodies against CD8, CD16, CD 19, CD36 and CD56 was prepared. The method of the invention results in 89% purity of CD4+ T cells, with a recovery of 64%.

A CD8+ T cell enrichment cocktail of tetrameric antibody complexes containing antibodies against CD4, CD16, CD 19, CD36 and CD56 was prepared. The method of the invention results in 80% purity of CD8+ T cells, with a recovery of 43%.

A B cell enrichment cocktail of tetrameric antibody complexes containing antibodies against CD2, CD3, CD16, CD36 and CD56 was prepared. The method of the invention results in 84% purity of B cells, with a recovery of 58%.

A NK cell enrichment cocktail of tetrameric antibody complexes containing antibodies against CD3, CD4, CD 19, CD36 and CD66b was prepared. The method of the invention results in 80% purity of NK cells, with a recovery of 50%.

Example 17

Immunorosetting Using Different Layering Media

This example demonstrates that the method of Example 2 can be modified by substituting different media for Ficoll-Hypaque in Step 4. The

density of Ficoll was 1.077 g/mL and the osmolarity was approximately 300 mOsm. Percoll and Iodixanol solutions were prepared with a density of 1.085 g/mL and an osmolarity of 280 mOsm. A B cell enrichment cocktail containing antibody complexes against CD2, CD3, CD16, CD36 and CD56 was prepared.

The results of B cell enrichments for two separate samples, shown in Table 16, demonstrate that the use of different layering media at a higher density can increase the recovery of B cells without lowering the B cell purity.

Example 18

Purging of T Cells Using Immunorosettes

This example demonstrates the removal of T cells from whole peripheral blood using the method described in Example 2. T cells purging cocktail of tetrameric antibody complexes to CD3 was prepared. The method of the invention resulted in 2.3 log depletion of CD3+ cells.

Example 19

Purging of B Cells Using Immunorosettes

This example demonstrates the removal of B cells from whole peripheral blood using the method described in Example 2. B cells purging cocktail of tetrameric antibody complexes to CD19 was prepared. The method of the invention resulted in 3.0 log depletion of CD19+ cells.

Example 20

Purging of Breast Carcinoma Cells Using Immunorosetting

This example demonstrates the removal of breast carcinoma cells from whole peripheral blood seeded with 1-5% CAMA breast carcinoma cells using the method described in Example 2. A purging cocktail of tetrameric antibody complexes containing anti-breast carcinoma antibodies 5E11 and BRST 1 was prepared. The results shown in Table 17 demonstrate the method of the invention results in 1.0-1.4 log depletion of breast carcinoma cells.

Example 21

Removal of Granulocytes from Previously Stored Whole Peripheral Blood

The density of granulocytes in samples of whole peripheral blood decreases with >24 hours of storage. Density separation methods commonly used to remove red cells and granulocytes from fresh whole blood do not efficiently remove granulocytes from stored blood samples. The sedimentation rate of stored granulocytes can be increased to allow efficient removal in a standard Ficoll density separation (1.077 g/mL) by immunorosetting. This example demonstrates the removal of granulocytes from stored (48 hour) whole peripheral blood using the method described in Example 2. A granulocyte depletion cocktail containing tetrameric antibody complexes against CD66b was prepared. The results, shown in Table 18, demonstrate that the method of the invention results in 1.8-2.6 log depletion of granulocytes.

Example 22

Positive Selection of Specific Cell Populations Using Immunorosetting

This example demonstrates the enrichment of CD8+ cells from whole peripheral blood using the positive selection method described in Example 5. A tetrameric antibody complex against CD8 was prepared. The method of the invention results in the enrichment of CD8+ cells as a percentage of the mononuclear cell fraction from 25% in the start to 32% in the pellet.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

TABLE 1

Antibodies used in Cell Separation

Antigen	Antibody	Source
CCR5	BLR-7	R&D, Minneapolis, MN
CD2	6F10.3 MT910	IMMUNOTECH, Marseille, France Dako, Carpinteria, CA
CD3	UCHT1 SK7	IMMUNOTECH, Marseille, France Becton Dickinson Immunocytometry, Mountain View, Calif.
CD4	13B8.2	Becton Dickinson Immunocytometry, Mountain View, Calif.
CD5	UCHT2	Serotec, Raleigh, NC
CD8	B911 OKT3	Becton Dickinson Immunocytometry, Mountain View, Calif. BioDesigns
CD10	ALB1	IMMUNOTECH, Marseille, France
CD11b	ICRF44	Pharmingen, San Diego, CA
CD14	MEM 15 MEM 18	Exbio, Praha, Czech Republic
CD15	DU-HL60-3	Sigma, St. Louis, MO
CD16	MEM 154 3G8 NKP15	Exbio, Praha, Czech Republic IMMUNOTECH, Marseille, France Becton Dickinson Immunocytometry, Mountain View, Calif.
CD19	J4.119 4G7 HD37	IMMUNOTECH, Marseille, France Becton Dickinson Immunocytometry, Mountain View, Calif. Dako, Carpinteria, CA
CD20	MEM97 L27	Exbio, Praha, Czech Republic Becton Dickinson Immunocytometry, Mountain View, Calif.
CD21	B-Ly4	Pharmingen, San Diego, CA
CD22	HIB22	Pharmingen, San Diego, CA
CD24	32D12 ALB9	Dr. Steinar Funderud, Institute for Cancer Research, Dept. of Immunology, Oslo, Norway IMMUNOTECH, Marseille, France
CD25	3G10	Caltaq, Burlingame, CA
CD27	1A4CD27	IMMUNOTECH, Marseille, France
CD29	Lia1.2	IMMUNOTECH, Marseille, France
CD33	D3HL60.251	IMMUNOTECH, Marseille, France
CD34	581	IMMUNOTECH, Marseille, France
CD36	FA6.152 IVC7	IMMUNOTECH, Marseille, France CLB, Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service
CD38	T16	IMMUNOTECH, Marseille, France

CD41	PI1.64 SZ22	Kaplan, 5th International Workshop on Human Leukocyte Differentiation Antigens IMMUNOTECH, Marseille, France
CD42a	Bebl	Becton Dickinson Immunocytometry, Mountain View, Calif.
CD45	J33 MEM28	IMMUNOTECH, Marseille, France Exbio, Praha, Czech Republic
CD45RA	8D2.2 L48	Craig et al. 1994, StemCell Technologies, Vancouver, Canada Becton Dickinson Immunocytometry, Mountain View, Calif.
CD45RO	UCHL1	Dako, Carpinteria, CA
CD56	T199 MY31	IMMUNOTECH, Marseille, France Becton Dickinson Immunocytometry, Mountain View, Calif.
CD66e	CLB/gran10	CLB, Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service
CD66b	B13.9 80H3	CLB, Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service IMMUNOTECH, Marseille, France
CD69	L78	BD Biosciences, San Jose, CA
CD71	My29	Zymed Laboratories, San Francisco, CA
CD124	S456C9	IMMUNOTECH, Marseille, France
HLADR	IMMU357.12	IMMUNOTECH, Marseille, France
IgA1	NiF2	IMMUNOTECH, Marseille, France
IgE	G7-18	Pharmingen, San Diego, CA
IgG	8A4	IMMUNOTECH, Marseille, France
TCRab	WT31	BD Biosciences, San Jose, CA
TCR gd	Immu510	IMMUNOTECH, Marseille, France

TABLE 2

Immunorosetting

Cocktails of Antibodies for Negative Selection of Human Cells

T Cell Enrichment
Anti-
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

Resting T Cell Enrichment
Anti-
HLA-DR and/or CD25, CD69
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

gd T Cell Enrichment
Anti-
abTCR
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

TABLE 2 (Cont'd)

abT Cell Enrichment
Anti-
gdTCR
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

CD4+ T Cell Enrichment
Anti-
CD8
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

Naïve CD4+ T Cell Enrichment
Anti-
CD8
CD45RO and/or CD29
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

TABLE 2 (Cont'd)

Memory CD4+ T Cell Enrichment
Anti-
CD8
CD45RA
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

Resting CD4+ T Cell Enrichment
Anti-
CD8
HLA-DR and/or CD25, CD69
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

CD4+ abT Cell Enrichment
Anti-
gdTCR
CD8
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

TABLE 2 (Cont'd)

TH1 CD4+ T Cell Enrichment
Anti-
CD8
CD124
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

TH2 CD4+ T Cell Enrichment
Anti-
CD8
CCR5
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

CD8+ T Cell Enrichment
Anti-
CD4
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

TABLE 2 (Cont'd)

Naïve CD8+ T Cell Enrichment
Anti-
CD4
CD45RO and/or CD29
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

Memory CD8+ T Cell Enrichment
Anti-
CD4
CD45RA
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

Resting CD8+ T Cell Enrichment
Anti-
CD4
HLA-DR and/or CD25, CD69, CD27
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

TABLE 2 (Cont'd)

CD8+ abT Cell Enrichment
Anti-
gdTCR
CD4
CD16 and/or CD66b, CD11b, CD15
CD19 and/or CD20, CD21, CD22, CD24, Ig
CD36 and/or CD14
and optionally anti-CD33, CD56, IgE, CD41

B Cell Enrichment
Anti-
CD2 and/or CD3, both CD4 and CD8
CD16 and/or CD66b, CD11b, CD15
CD36 and/or CD14
and optionally anti-CD33, CD56, CD41

NK Cell Enrichment
Anti-
CD3
CD66b and/or CD15
CD19 and/or CD20, CD21, CD22, CD24
CD36 and/or CD14
and optionally anti-CD33, CD4, IgE, CD41

Monocyte Enrichment
Anti-
CD2 and/or CD3, CD5
CD19 and/or CD20, CD21, CD22, CD24
CD66b and/or CD16
and optionally anti-CD8, CD56

TABLE 2 (Cont'd)

Dendritic Cell Enrichment
Anti-
CD3
CD14
CD16
CD19
CD34
CD56
CD66b

Basophil Enrichment
Anti-
CD2
CD3
CD14
CD15
CD16
CD19
CD24
CD34
CD36
CD56
CD45RA

TABLE 2 (Cont'd)

Progenitor Enrichment
Anti-
CD2 and/or CD3
CD16 and/or CD66b
CD19 and/or CD24
CD14
and optionally anti- CD56, CD10, CD45RA, CD38, CD36, CD33, CD71

Erythroid Progenitor Enrichment
Anti-
CD2 and/or CD3
CD16 and/or CD66b
CD19 and/or CD24
CD14
CD45RA
CD33
CD10
and optionally anti- CD56

TABLE 2 (Cont'd)

Myeloid Progenitor Enrichment
Anti-
CD2 and/or CD3
CD16 and/or CD66b
CD19 and/or CD24
CD14
CD71
CD10
and optionally anti- CD56

Megakaryocyte Progenitor Enrichment
Anti-
CD2 and/or CD3
CD16 and/or CD66b
CD19 and/or CD24
CD14
CD45RA
CD10
and optionally anti- CD56

Epithelial Tumor Cell Enrichment
Anti-
CD45
CD66b
and optionally CD36, CD2, CD3, CD14, CD16, CD19, CD38, CD56, CD66e

TABLE 3

Antibodies Recognizing Non-Hematopoietic Antigens Expressed on Epithelial Tumor Cells

Specificity	Antibody	Antigen	Supplier/Developer
Epithelial cell markers	BerEp4	ESA, (Epithelial Specific Antigen) (also known as HEA)	DAKO
	HEA125	ESA	Serotec, Cymbus, Pierce, RDI, Biodesign
	VU-1D9	ESA	Cymbus
	GP1.4	EMA, (Epithelial Membrane Antigen)(also known as PEM / Episialin, a sialomucin)	IMMUNOTECH, Marseille, France
	VU-4H5	EMA	Neomarkers
	MC.5	EMA	Biogenex, also Biodesign
	B24.1	EMA	Biomeda
	E29	EMA	DAKO
	H11	EGFR	DAKO
	RAR9941	epithelial glycoprotein	Baxter, Germany
	RAR9948	epithelial glycoprotein	Baxter, Germany
Carcinoma (breast, cervical, ovarian, lung, endometrial)	CU-18	BCA 225 (Breast carcinoma associated antigen)	ID Labs
Carcinoma	115D8	Carcinoma associated antigen	Biogenex, Biodesign
Adenocarcinomas	B72.3	TAG-72 (Tumour associated glycoprotein)	ID Labs, Biogenex, Signet
Adenocarcinomas, mammary & lung carcinomas	B6.2	Unknown, breast cancer marker	Biogenex
Breast Carcinoma	5E11	unknown, breast carcinoma	STI
	6E7	unknown, breast carcinoma	STI
	H23A	unknown, breast carcinoma	STI
	CA27.29	MAM-6, mucin	Cedarlane
	SM-3	milk mucin core antigen	Cymbus, Biodesign,

			Imperial Cancer Research Fund
	DF3	CA 15-3 (breast tumour marker)	ID Labs
	552	CA 15-3	Biodesign
	695	CA 15-3	Biodesign
	RAR9938	c-erb B2	Baxter, Germany
	C13B5	c-erb B2	IMMUNOTECH, Marseille, France, also Biogenex
Lung	MOC-1	Small cell lung carcinoma	ICN Biomed, also Biodesign
	MOC-21	Small cell lung carcinoma	ICN Biomed, also Biodesign
	MOC-31	Small cell lung carcinoma	ICN Biomed, also Biodesign
	MOC-32	Small cell lung carcinoma	ICN Biomed, also Biodesign
	MOC-52	Small cell lung carcinoma	ICN Biomed, also Biodesign
	TFS-4	Small cell lung carcinoma	Biodesign
Melanoma	NKI/C3	Melanoma associated antigen	ICN Biomed, also Biodesign
	NKI/M6	Melanoma associated antigen	Biodesign
	PAL-MI	Melanoma associated antigen	ICN Biomed, also Biodesign
	HMB45	Melanoma cells	Biodesign
Ovarian tumour	185	CA-125 (ovarian tumour marker)	ICN Biomed, also Biodesign
	OV-632	Ovarian cancinoma marker	ICN Biomed, also Biodesign
Gastro-Intestinal Cancer	CA 19-9	GI tumour marker	ICN Biomed, also Biodesign
	CA 242	GI cancer	BioDesign
Renal Cell Carcinoma	RC38	Renal Cell Carcinoma	Biodesign
Ewing's Sarcoma	O13		Signet
Ewing's Sarcoma	CC49	on human adenocarcinomas	Signet
Neuroblastoma	UJ13A	unknown	Hurko and Walsh (1983) Neurology 33:734
	UJ181.4	unknown	"
	UJ223.8	unknown	"

	UJ127.11	unknown	"
	5.1.H11	unknown	"
	390,459	unknown	R.C. Seeger, L. A. Children's Hospital, Calif.
	BA-1.2	unknown	"
	HSAN 1.2	unknown	Reynolds and Smith (1982) Hybridomas in Cancer p235

TABLE 4

T Cell Enrichment - Immunorosetting Using Ficoll

Purity	mean	95
	SD	4
	n	19
Recovery	mean	46
	SD	12
	n	19

SD = Standard deviation from the mean

Purity = % CD3+ cells

Recovery = Recovery of CD3+ cells

TABLE 5

CD8+ T Cell Enrichment - Immunorosetting Using Ficoll

Cocktail	n	% Purity ±1SD	% Recovery ± 1SD
CD4, CD16, CD19, CD36, CD56	19	76±8	44±19
CD4, CD16, CD19, CD36, CD56, IgE	5	81±4	45±37*

SD = Standard Deviation from the mean

Purity = % CD8+ cells

Recovery = % Recovery of CD8+ cells

* n = 4

TABLE 6

CD4+ T Cell Enrichment - Immunorosetting Using Ficoll

Cocktail	n	% Purity ± 1SD	% Recovery ± 1SD
CD8, CD16, CD19, CD36, CD56	19	89±4	57±22
CD8, CD16, CD19, CD36, CD56, IgE	7	93±3	46±10*

SD = Standard Deviation from the mean

Purity = % CD4+ cells

Recovery = % Recovery of CD4+ cells

* n = 5

TABLE 7

B Cell Enrichment - Immunorosetting Using Ficoll

Cocktail	n	% Purity ±1SD	% Recovery ± 1SD
CD2, CD3, CD16, CD36, CD56	22	72±15	61±27
CD2, CD3, CD16, CD36, CD56, IgE	5	88±7	43±18

SD = Standard Deviation from the mean

Purity = % CD19+ cells

Recovery = % Recovery of CD19+ cells

TABLE 8

NK Cell Enrichment - Immunorosetting Using Ficoll

Cocktail	n	% Purity ±1SD	% Recovery ± 1SD
CD3, CD4, CD19, CD66b, CD36	15	74±10	44±19
CD3, CD4, CD19, CD66b, CD36, IgE	6	88±4	27±20

SD = Standard Deviation from the mean.

Purity = % CD56+ cells

Recovery = % recovery CD56+ cells

TABLE 9

Enrichment of CD34+ cells from Whole Cord Blood - Immunorosetting Using Ficoll

Cocktail	n	% Purity ±1SD	% Recovery ±1SD
Progenitor Enrichment	15	29 ± 9	53 ± 29
Debulking	8	5 ± 1	45 ± 20

Purity = % CD34+ cells

Recovery = % recovery CD34+ cells

SD = Standard Deviation from the mean

TABLE 10

Monocyte Enrichment - Immunorosetting Using Ficoll

Cocktail	n	% Purity ±1SD	% Recovery ± 1SD
CD2, CD3, CD19, CD56, CD66b	8	71±7	63±28
CD2, CD3, CD19, CD56, CD66b, CD8	5	76±1.5	65±28
CD2, CD3, CD19, CD56, CD66b, IgE	6	77±4	58±24
CD2, CD3, CD19, CD56, CD66b, IgE, CD8	4	76±3	64±26
CD2, CD3, CD19, CD56, CD66b, CD16	1	76	64
CD2, CD3, CD19, CD56, CD66b, CD20	1	73	41

Purity = %Purity CD14+ cells

Recovery = %Recovery CD14+ cells

TABLE 11

Antibody Composition of Tumor Enrichment Cocktails

5

Cocktail	Antibodies in Cocktail
CD45 alone	CD45
CD45 and CD66b	CD45, CD66b
CD45, CD66b and CD36	CD45, CD66b, CD36
Extensive cocktail	CD45, CD2, CD16, CD19, CD36, CD38, CD66b

TABLE 12

Enrichment of CAMA Breast Cancer Cells from Whole Blood

Starting frequency (CAMA)	1/103	1/104	1/105	1/103	1/104	1/105	1/103	1/104	1/105
	% Purity of CAMA Cells			Log Enrichment of CAMA Cells			% Recovery of CAMA Cells		
Enriched									
CD45 alone	4±2 (n=4)	5±2 (n=7)	0.5±0.4 (n=3)	1.4±0.3 (n=4)	2.2±0.3 (n=7)	2.3±0.4 (n=3)	10±3 (n=4)	26±7 (n=5)	55±36 (n=2)
CD45 and 66b	27±4 (n=6)	3.2±0.6 (n=6)	0.5±0.1 (n=5)	2.4±0.1 (n=6)	2.5±0.1 (n=6)	2.7±0.1 (n=5)	15±2 (n=6)	12±1 (n=5)	22±4 (n=5)
Extensive Cocktail	65±8 (n=9)	26±8 (n=9)	3±1 (n=6)	2.8±0.1 (n=9)	3.2±0.2 (n=9)	3.2±0.3 (n=6)	38±8 (n=7)	49±14 (n=5)	33±7 (n=5)

TABLE 13

The Effects of Anti-CD36 Antibody in the Epithelial Tumour Enrichment Cocktail

Cocktail	% Purity CAMA Cells	% Recovery CAMA Cells	Log Enrichment CAMA cells
none – Ficoll alone	0.6	20	0.6
CD45, CD66b	58	11	2.6
CD45, CD66b, CD36	77	18	2.7
Extensive Cocktail	90	17	2.8

TABLE 14

T Cell Enrichment - Immunorosetting Using Ficoll

	n	Cocktail with CD14 ±1SD	Cocktail with CD36 ±1SD
Purity	3	80±10	94±5
Recovery	3	56±12	42±10

SD = Standard Deviation from the mean

Purity = %Purity CD3+ cells

Recovery = %Recovery CD3+ cells

TABLE 15

Immunorosetting Using Hetastarch/Iodixanol Mixture

Cell Type Enriched	Purity			Recovery		
	mean	SD	n	mean	SD	n
T cells	95%	3%	3	61%	9%	3
CD4+ cells	89%	5%	2	64%	5%	2
CD8+ cells	80%	8%	2	43%	1%	2
B cells	84%	8%	5	58%	26%	5
NK cells	80%	15%	4	50%	23%	4

SD = Standard Deviation from the mean

Purity = % Purity desired cell type (T cells = CD3+ cells, CD4+ cells, CD8+ cells, B cells = CD19+ cells, NK cells = CD56+ cells)

Recovery = % Recovery of desired cells

TABLE 16

Immunorosetting Using Different Layering Media

B Cell Enrichment

Media	Ficoll	Percoll	Iodixanol
Sample 1 (in triplicate)			
Purity ± 1SE	82±2.9	81±1.4	86±2.7
Recovery ± 1SE	78±6.0	110±3	104±10
Sample 2 (in triplicate)			
Purity ± 1SE	71±1.2	77±1.5	81±2.4
Recovery ± 1SE	49±8	78±3	64±1

SE = Standrad Error of the mean

Purity = % Purity of CD19+ cells

Recovery = % Recovery of CD19+ cells

TABLE 17

Puging of Breast Carcinoma Cells Using Immunorosetting

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	Sample 1	Sample 2
Log depletion of CAMA cells	1.4, 1.4	1.1, 1.0

TABLE 18

Removal of Granulocytes from Stored Whole Peripheral Blood

	Immunorosetting	Ficoll alone
% Granulocytes in light density fraction	1.1, 1.4, 0.7, 0.4	20.9, 18.0

Immunorosetting = Method outlined in Example 2, with depletion cocktail containing anti-CD66b

Ficoll alone = Standard Ficoll density separation without immunorosetting

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